Research

Retracted: Hyaluronan Activation of the Nlrp3 Inflammasome Contributes to the Development of Airway Hyperresponsiveness

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BACKGROUND: The role of the Nlrp3 inflammasome in nonallergic airway hyperresponsiveness (AHR) has not previously been reported. Recent evidence supports both interleukin (IL) 1β and short fragments of hyaluronan (HA) as contributors to the biological response to inhaled ozone.

OBJECTIVE: Because extracellular secretion of IL-1β requires activation of the inflammasome, we investigated the role of the inflammasome proteins ASC, caspase1, and Nlrp3 in the biological response to ozone and HA.

METHODS: C57BL/6J wild-type mice and mice deficient in ASC, caspase1, or Nlrp3 were exposed to ozone (1 ppm for 3 hr) or HA followed by analysis of airway resistance, cellular inflammation, and total protein and cytokines in bronchoalveolar lavage fluid (BALF). Transcription levels of *IL-1*β and *IL-18* were determined in two populations of lung macrophages. In addition, we examined levels of cleaved caspase1 and cleaved IL-1β as markers of inflammasome activation in isolated alveolar macrophages harvested from BALF from HA-treated mice.

RESULTS: We observed that genes of the Nlrp3 inflammasome were required for development of AHR following exposure to either ozone or HA fragments. These genes are partially required for the cellular inflammatory response to ozone. The expression of *IL-1*β mRNA in alveolar macrophages was up-regulated after either ozone or HA challenge and was not dependent on the Nlrp3 inflammasome. However, soluble levels of IL-1β protein were dependent on the inflammasome after challenge with either ozone or HA. HA challenge resulted in cleavage of macrophage-derived caspase1 and IL-1β, suggesting a role for alveolar macrophages in Nlrp3-dependent AHR.

CONCLUSIONS: The Nlrp3 inflammasome is required for the development of ozone-induced reactive airways disease.

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Ambient ozone is a highly reactive oxidant associated with increased morbidity and mortality in human populations (Bell et al. 2004, 2006; Ito et al. 2005). Despite effective regulatory oversight to reduce ambient levels of ozone (Samet 2011), current evidence suggests that adverse health effects may occur at exposures below current regulatory standards (Berman et al. 2012; Kim et al. 2011). Furthermore, changes in global climate are anticipated to increase both ambient levels of ozone and associated adverse health effects (Chang et al. 2010). Inhalation of ozone can immediately cause lung injury, inflammation, and decrements in lung function (Foster et al. 2000; Kehrl et al. 1987; Koren et al. 1989). The delayed responses to ozone include induction of asthma-like phenotypes with compromises in epithelial barrier function and development of airway hyperresponsiveness (AHR) (Que et al. 2011). For this reason, we believe that fundamental insight into the mechanisms that regulate the biological response to ozone could have broad implications to our understanding of the pathogenesis of airways diseases, including asthma and chronic obstructive pulmonary disease (COPD).

The biological response to ozone appears to be dependent on activation of the innate immune system (reviewed by Al-Hegelan et al. 2011; Peden 2011). Components of the response to ozone requires activation of the surface receptor toll-like receptor 4 (TLR4) (Hollingsworth et al. 2004; Kleeberger et al. 2001; Williams et al. 2007). More recent data have demonstrated that ozone-induced production of short fragments of hyaluronan (HA) (Garantziotis et al. 2009) activates the TLR4–CD44 surface receptor complex and contributes to AHR (Garantziotis et al. 2010; Taylor et al. 2007). Interestingly, short fragments of HA are also detected in bronchoalveolar lavage (BAL) fluid (BALF) from patients with COPD (Dentener et al. 2005) and allergic asthma (Liang et al. 2011; Sahu and Lynn 1978). In our previous studies (Garantziotis et al. 2010; Li et al. 2011), the response to either ozone or HA fragments were also closely associated with the levels of interleukin (IL)-1β in the BALF. Previous work supports a central role for IL-1–dependent signaling in the biological response to ozone (Johnston et al. 2007; Park et al. 2004; Verhein et al. 2008; Wu et al.

2008). Although the specific mechanism by which IL-1β contributes to AHR remains unknown, IL-1β is recognized to increase both cyclooxygenase (COX)-2 expression and prostaglandin E_2, which results in decreased β-adrenergic responsiveness of smooth muscle cells, providing a link to AHR (Moore et al. 2001). However, the mechanisms leading to IL-1β activation in the context of either ozone exposure or reactive airways disease remains poorly understood.

The inflammasome protein complex is an important regulator of IL-1β and IL-18 maturation and release into the extracellular space (reviewed by Hoffman and Brydges 2011; Jin and Flavell 2010). The inflammasome complex consists of the protein pro-caspase1 and may include the adaptor molecule ASC, as well as specific members of the nucleotide-binding domain leucine-rich repeat-containing (NLR) family. Inflammasome activation leads to caspase1-mediated cleavage and activation of the proinflammatory cytokines pro-IL-1β and pro-IL-18. Yamasaki et al. (2009) recently demonstrated that HA-induced IL-1β release is dependent on the Nlrp3 inflammasome in a model of sterile skin injury. Recent work in macrophages showed that oxidant stress can prime the Nlrp3 inflammasome (Bauernfeind et al. 2011). On the basis of these observations and the recognized role of HA in the response to ozone, we hypothesized that HA activation of the Nlrp3 inflammasome would contribute to the airway response to ozone.

Materials and Methods

Mice. Wild-type (WT) C57BL/6J mice were purchased from Jackson Laboratory (Bar Harbor, ME, USA). Mice deficient in caspase1 (caspase1$^{-/-}$) were generously provided by F. Sutterwala and R. Flavell (Sutterwala et al. 2006). ASC-deficient (ASC$^{-/-}$) and Nlrp3-deficient (Nlrp3$^{-/-}$) mice were generously provided by Genentech Inc. (San Francisco, CA, USA). Male mice, 6–8 weeks of age, were used in experiments. Animals were treated humanely with due consideration to alleviation of distress and discomfort. Experimental protocols were approved by the Institutional Animal Care Committee at Duke University Medical Center and performed in accordance with established guidelines (National Research Council 1996).

Ozone exposure. Mice were exposed to either filtered air or ozone (1 ppm × 3 hr) in a Hinner-style chamber as previously described (Hollingsworth et al. 2004). The ozone concentrations used are based on similar biological responses observed in human exposure studies and in published deposition fraction data for ozone in rodent models (Hatch et al. 1994; Wiester et al. 1988). The ozone concentration in the chamber was monitored continuously by an ultraviolet light photometer (model 400E; Teledyne Technologies Inc., Thousand Oaks, CA, USA). WT mice were phenotyped 3, 6, 12, 24, 48, and 72 hr after ozone exposure. Twenty-four hours after exposure, we observed the phenotype of C57BL/6J and inflammasome-deficient mice based on the level of cellular inflammation, HA in BALF, and the transcription level of IL-1β. In blocking experiments, C57BL/6J mice were anesthetized with isoflurane; then 0.22 mg hyaluronic acid-binding peptide (HABP) or scrambled-binding peptide (GenScript, Piscataway, NJ, USA) were oropharyngeally instilled into the lung immediately before ozone exposure.

HA challenge. Sterile, endotoxin-free high-molecular-weight HA (Healon GV; Abbott Medical Optics, Santa Ana, CA, USA) was reconstituted to 0.5 mg/mL in 0.02 M acetate, 0.15 M sodium chloride, pH 6.0. For the production of low-molecular-weight HA, high-molecular-weight HA was sonicated 3 sec three times on ice. Sizes of HA fragments were confirmed by agarose gel electrophoresis. For *in vivo* experiments, 50 μL HA (25 μg/mouse) or vehicle were instilled intratracheally into isoflurane-anesthetized mice. This HA dose was sufficient to significantly induce AHR (Garantziotis et al. 2009). AHR was measured invasively 2-hr later. The selection of HA treatment time point was based on HA time course experiments.

Airway physiology. We performed direct measurements of airway response to methacholine as previously reported (Garantziotis et al. 2009). Briefly, mice were ventilated with a computer-controlled small animal ventilator (FlexiVent; SCIREQ, Montreal, Quebec, Canada), and measurements of respiratory mechanics were made by the forced oscillation technique. Mice were challenged with aerosolized methacholine at 0, 10, 25, or 100 mg/mL, and airway response was determined by resistance measurements every 30 sec for 5 min. Total lung resistance (R_T) measurements (given in centimeters of water per milliliter of gas per second; R_T cm H_2O/mL/sec) were averaged at each dose and graphed along with the initial baseline measurement.

BAL. BAL was performed as described previously (Garantziotis et al. 2009). Briefly, immediately after pulmonary function measurements, mice were euthanized with CO_2, and the lungs were exposed and inflated with 0.9% NaCl three times to a pressure of 25 cm H_2O. Cell counting was performed using a hemocytometer, and differentials were performed using hematoxylin and eosin–stained cytospins. Cytokines/chemokines [IL-1α, IL-1β, IL-6, KC (cytokine-induced neutrophil chemoattractant), MCP-1 (monocyte chemoattractant protein-1), IL-17, and TNF-α (tumor necrosis factor α)] were determined by Luminex (Bio-Rad, Hercules, CA, USA) using reagents from Millipore (Billerica, MA, USA). IL-18 and C3a were detected by ELISA (Bender MedSystems, Vienna, Austria; Kamiya Biomedical Company, Seattle, WA, USA). Total protein in BALF was detected by Lowry Assay (Bio-Rad), and HA level in BALF was measured by ELISA (Echelon, Salt Lake City, UT, USA).

Alveolar macrophage isolation. Alveolar macrophages were harvested by BAL with 0.9% NaCl/0.5 mM EDTA buffer. Briefly, a total of 10 fills of lavage fluid was collected. After centrifugation at 1,500 rpm for 10 min, the cell pellet obtained was used for RNA analysis. We achieved 98% macrophages with this technique, as detected by cytospin.

Whole-lung macrophage isolation. After taking airway physiology measurements and collecting BAL, the lungs were removed from the mouse and placed into a tissue culture dish. They were minced and digested in Hank's balanced salt solution with 1 mg/mL collagenase A and 0.2 mg/mL of DNase1 (both from Sigma Chemical Co., St. Louis, MO, USA) for 1 hr at 37°C. The digestion solution was passed through a 70-μm mesh strainer and centrifuged at 1,600 rpm for 20 min at room temperature over an 18% nycodenz (Accurate Chemical Co.) cushion. After red blood cell lysis, low density cells were collected and then washed twice. We achieved 87% macrophages by cytospin using this technique. The cell pellet was retained for RNA analysis.

Real-time polymerase chain reaction (PCR). Total RNA was isolated using the RNAeasy kit (Invitrogen, Carlsbad, CA, USA). Samples were treated with DNase treatment and removal reagents to remove DNA contamination (Ambion Inc., Austin, TX, USA). RNA samples were then reverse-transcribed into cDNA using SuperScript II RT (Invitrogen) following the manufacturer's protocol. Real-time PCR was performed using the SYBR green detection system (Applied Biosystems, Foster City, CA, USA). Primer sequences used for *IL-1* and *IL-18* amplification are as follows: *IL-1*β forward: 5′-CTGTGTCTT TCCCGTG GACC-3′, reverse 5′-CAGCTCATATG GGTCCGACA-3′; *IL-18* forward: 5′-CACATGCGCCTTGTGATGAC-3′, reverse 5′-TGCAGCCTCG GGTAT TCTG-3′; 18S forward: 5′-GCTGCTG GCACCAGACTT-3′, reverse 5′-CGGCTAC CACATCCAAGG-3′. After ΔΔCt calculation, all results were normalized to 18S ribosomal RNA internal controls and expressed as fold increase of RNA expression compared with the vehicle control.

Immunoblot analysis. BALF from five mice per group was pooled into one sample and centrifuged to form an alveolar macrophage cell pellet, which was then centrifuged and dissolved in RIPA lysis buffer containing proteinase inhibitors. The protein concentration was determined using the DC Protein Assay (Bio-Rad), and 20 μg protein per sample was mixed with sample loading buffer and boiled for 5 min. Samples were separated by 4–20% SDS-PAGE and transferred onto polyvinylidene fluoride membranes (Bio-Rad). Immunoblots were blocked with 5% milk in Tris-buffered saline (TBS)/0.1% Tween 20 for 1 hr at room temperature and incubated overnight at 4°C with rabbit polyclonal anti-mouse caspase1 p10 (1:1,000; Santa Cruz Biotechnology, Santa Cruz, CA, USA), goat polyclonal anti-mouse IL-1β (1:2,000; R&D Systems, Minneapolis, MN, USA), or goat polyclonal anti-mouse GAPDH (1:1,000; Santa Cruz Biotechnology) in 3% milk/TBS/0.1% Tween 20. Membranes were incubated with the secondary antibody— goat anti-rabbit IgG (1:2,000) or donkey anti-goat IgG (1:2,000)—in 3% milk/TBS/0.1% Tween 20 at room temperature for 1 hr. Bands were detected using the ECL Plus Western Blotting Detection Kit (GE Healthcare, Piscataway, NJ, USA).

Statistics. Data are representative of at least two replicate experiments and are expressed as mean ± SE. We used GraphPad software (San Diego, CA, USA) for statistical analyses. We used analysis of variance to determine significant differences among multiple groups with one variant, and every two groups were then compared by the Neuman–Keuls method. The Student *t*-test was used for comparisons between two groups. A two-tailed *p*-value < 0.05 was considered statistically significant.

Results

Significant biological response in the lung was observed 24 hr after ozone inhalation. We examined the airway response to ozone (1 ppm for 3 hr) in WT mice over a 72-hr time course. We observed a significant increase in the total number of inflammatory cells 24–72 hr after ozone exposure (Figure 1A); BALF differential cell counts revealed an increase in the numbers of macrophages and neutrophils (Figure 1B,C). Cellular inflammation peaked 24 hr after ozone exposure and maintained a high level until 72 hr. The level of BALF protein peaked at 12–48 hr and declined to near baseline at 72 hr (Figure 1D). We detected BALF HA levels to examine extracellular matrix injury during ozone-induced acute airway inflammation. The level of HA in BALF increased at 24 hr, peaked at 48 hr, and declined by 72 hr after ozone exposure (Figure 1E). We examined the transcription level of *IL-1*β and *IL-18* and found that *IL-1*β mRNA in alveolar macrophages was up-regulated at 6 hr, and this level remained at a similar level throughout the 72-hr time course (Figure 1F). Expression of *IL-18* mRNA in alveolar macrophages was increased at 24 hr and peaked at 72 hr (Figure 1G). On the basis of previous

epidemiological data and this time course, we selected the 24-hr time point to conduct subsequent studies.

The Nlrp3 inflammasome regulates the inflammatory response and AHR after ozone exposure. We exposed WT, ASC^{−/−}, caspase1^{−/−}, and Nlrp3^{−/−} mice to either filtered air or ozone (1 ppm for 3 hr). In a methacholine challenge 24 hr after exposure, we observed that ozone-exposed WT mice developed enhanced sensitivity to methacholine compared with air-exposed controls. ASC^{−/−}, caspase1^{−/−}, and Nlrp3^{−/−} mice were protected from enhanced AHR after ozone exposure, with values for R_T similar to those of air-exposed WT animals (Figure 2A). We characterized the level of inflammatory cell influx into the airspace after ozone exposure in each mouse strain (Figure 2B). As we anticipated, the number of total cells, macrophages, and neutrophils in BALF from ozone-exposed WT mice were higher than those from air-exposed animals. However, we observed significantly reduced numbers of macrophages and neutrophils in ASC^{−/−}, caspase1^{−/−}, and Nlrp3^{−/−} mice compared with ozone-exposed WT animals.

When we measured the level of total protein in BALF as a marker of lung injury after ozone inhalation, we found

that inflammasome-deficient animals had significantly lower levels of total protein in the BALF than ozone-exposed WT mice (Figure 2C). To measure cytokine expression directly regulated by the Nlrp3 inflammasome in response to ozone inhalation, we measured transcription levels of *IL-1*β and *IL-18* in alveolar macrophages and soluble levels of IL-1β and IL-18 in BALF at 24 hr after exposure. We observed up-regulation of *IL-1*β and *IL-18* mRNA in alveolar macrophages in ozone-exposed mice compared with air-exposed controls; this respose was not dependent on the Nlrp3 inflammasome (Figure 3A,B). In addition, we observed no differences in mRNA expression of *IL-1*β and *IL-18* in whole lung macrophages after ozone exposure [see Supplemental Material, Figure S1A,B (http://dx.doi.org/10.1289/ehp.1205188)]. The levels of secreted IL-1β and IL-18 protein in BALF from ozone-exposed WT mice were significantly higher than those in BALF from ozone-exposed ASC^{−/−}, caspase1^{−/−}, and Nlrp3^{−/−} mice (Figure 3C,D). We also observed a broad impact of inflammasome activation on additional proinflammatory cytokines and mediators previously associated with the biological response to ozone (see Supplemental Material, Figure S2A–G).
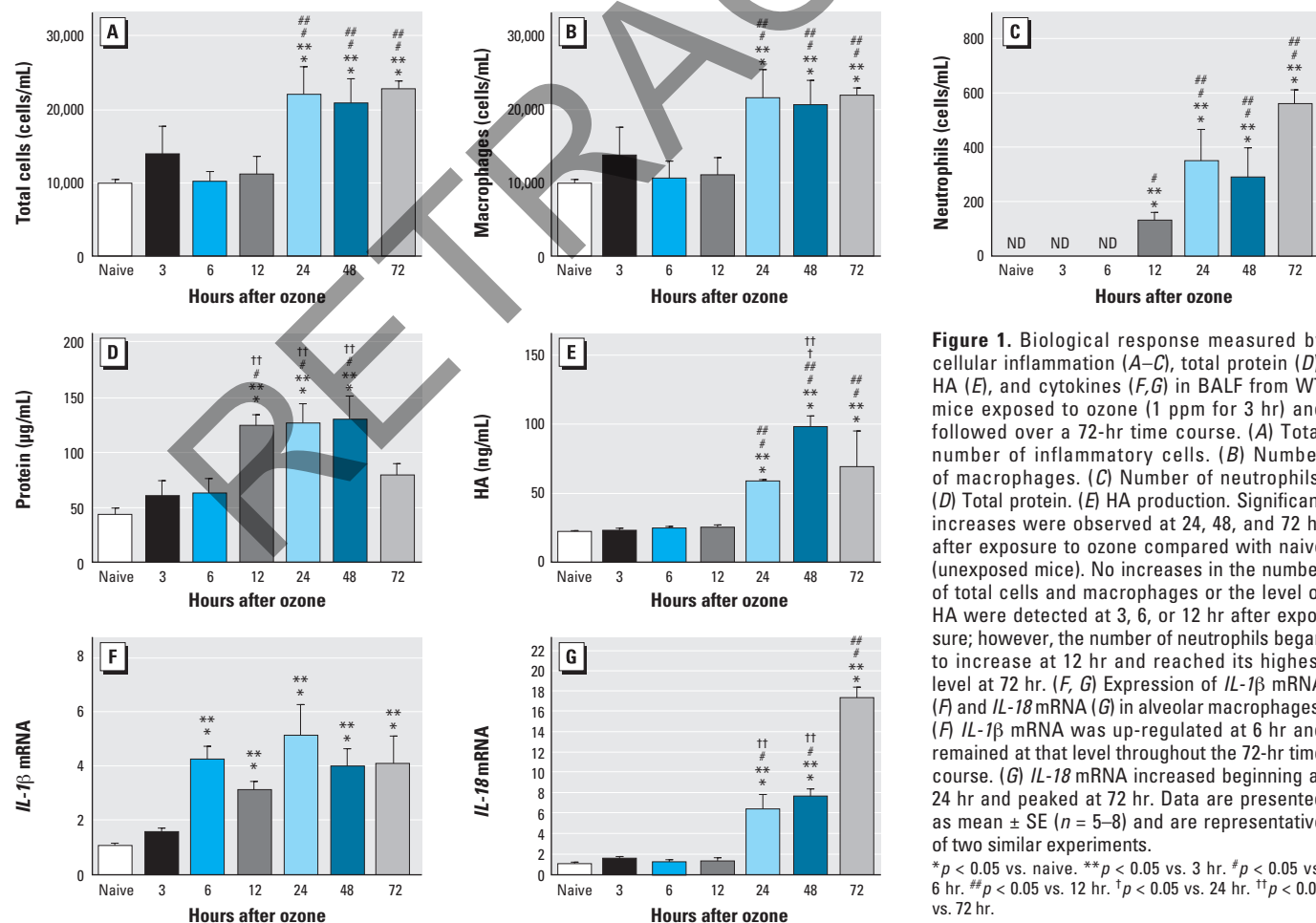

Figure 1. Biological response measured by cellular inflammation (*A*–*C*), total protein (*D*), HA (*E*), and cytokines (*F,G*) in BALF from WT mice exposed to ozone (1 ppm for 3 hr) and followed over a 72-hr time course. (*A*) Total number of inflammatory cells. (*B*) Number of macrophages. (*C*) Number of neutrophils. (*D*) Total protein. (*E*) HA production. Significant increases were observed at 24, 48, and 72 hr after exposure to ozone compared with naive (unexposed mice). No increases in the number of total cells and macrophages or the level of HA were detected at 3, 6, or 12 hr after exposure; however, the number of neutrophils began to increase at 12 hr and reached its highest level at 72 hr. (*F, G*) Expression of *IL-1*β mRNA (*F*) and *IL-18* mRNA (*G*) in alveolar macrophages. (*F*) *IL-1*β mRNA was up-regulated at 6 hr and remained at that level throughout the 72-hr time course. (*G*) *IL-18* mRNA increased beginning at 24 hr and peaked at 72 hr. Data are presented as mean ± SE (*n* = 5–8) and are representative of two similar experiments.
*$p < 0.05$ vs. naive. **$p < 0.05$ vs. 3 hr. #$p < 0.05$ vs. 6 hr. ##$p < 0.05$ vs. 12 hr. †$p < 0.05$ vs. 24 hr. ††$p < 0.05$ vs. 72 hr.

Ozone-exposed animals deficient in ASC and caspase1, and to a lesser extent in Nlrp3, had reduced levels of IL-1α, IL-6, TNF-α, and KC in BALF compared with WT animals. MCP-1 was completely dependent on Nlrp3; IL-17 was completely dependent on caspase1, Nlrp3, and partially dependent on ASC; and C3a was partially dependent on Nlrp3 inflammasome.

HA contributes to the increased level of secreted IL-1β in BALF after ozone exposure. Similar to observations in WT animals, the levels of soluble HA in BALF from ASC-, caspase1-, and Nlrp3-deficient animals were increased 24 hr after inhalation of ozone, but values were not significantly different compared with ozone-exposed WT animals (Figure 3E). To further identify the role of HA in ozone-induced inflammasome activation, we pretreated WT animals with HABP, which reduces the level of HA in the lungs (Garantziotis et al. 2009), immediately before ozone exposure and then measured the level of secreted IL-1β in BALF. We found that HABP pretreatment partially blocked the production of BALF IL-1β (Figure 3F); this supports the contribution of HA to ozone-induced release of soluble IL-1β.

Significant biological response in the lung is observed after direct HA challenge. Because HA was previously reported to be functionally relevant (Garantziotis et al. 2009), we instilled HA (25 μg/mouse) or vehicle intratracheally

into isoflurane-anesthetized WT mice. We then characterized the biological responses at 1, 2, and 6 hr after HA challenge. We found no increase in numbers of total cells, macrophages, or neutrophils in BALF until 6 hr after the challenge (Figure 4A–C). No increase in BALF protein was detected after HA challenge at any of the time points (Figure 4D). However, *IL-1β* mRNA in alveolar macrophages was up-regulated at both 1 hr and 2 hr after HA challenge compared with vehicle controls, but the level decreased to near baseline at 6 hr (Figure 4E). We observed no significant difference in the transcription level of *IL-18* after HA challenge compared with vehicle at any of the three time points (Figure 4F). Based on the data of *IL-1β* mRNA and the physiological response (Garantziotis et al. 2009, 2010; Li et al. 2011), we selected 2 hr after HA challenge for subsequent experiments.

The Nlrp3 inflammasome is required for pulmonary response to HA fragments. To determine the role of HA activation of the inflammasome in reactive airways disease, we directly challenged animals to short fragments of HA. As previously reported (Garantziotis et al. 2009, 2010; Li et al. 2011), WT animals developed AHR 2 hr after challenge with HA but not with vehicle (Figure 5). The physiological response, as measured by methacholine sensitivity after treatment with HA fragments,

was completely dependent on ASC, caspase1, and Nlrp3. However, similar to WT animals, HA challenge in ASC$^{-/-}$, caspase1$^{-/-}$, and Nlrp3$^{-/-}$ mice had no effect on either the number of cells [see Supplemental Material, Figure S3A (http://dx.doi.org/10.1289/ehp.1205188)] or the level of total protein (see Supplemental Material, Figure S3B) in BALF at this time point. After HA treatment, *IL-1β* mRNA in alveolar macrophages was up-regulated in a manner independent of the Nlrp3 inflammasome (Figure 6A), similar to that observed after ozone exposure; the release of soluble IL-1β was completely dependent on ASC and caspase1, and partially dependent on Nlrp3 (Figure 6C). However, we observed no detectable increases in *IL-18* mRNA expression in alveolar macrophages or soluble IL-18 in BALF after direct challenge with HA (Figure 6B,D). The increases of *IL-1β* and *IL-18* mRNA observed after HA challenge was not detected in whole-lung macrophages (see Supplemental Material, Figure S1C–D). Moreover, the release of IL-1α, IL-6, MCP-1, TNF-α, and KC were dependent on both ASC and caspase1 and partially dependent on Nlrp3; IL-17 was partially dependent on ASC and caspase1 but not on Nlrp3. C3a was dependent on ASC and caspase1 and partially dependent on Nlrp3 after HA fragment challenge (see Supplemental Material, Figure S4A–G).
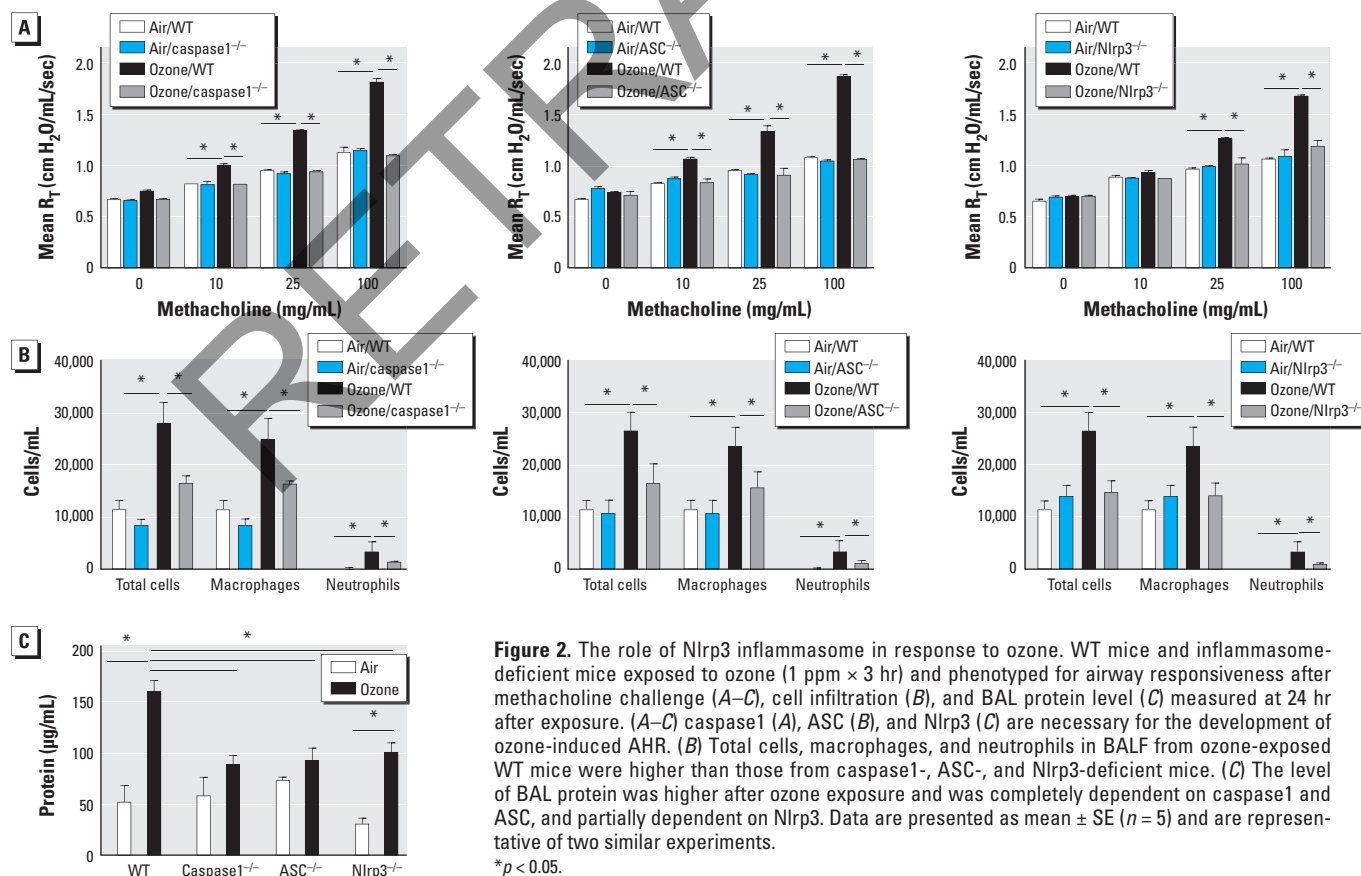

Figure 2. The role of Nlrp3 inflammasome in response to ozone. WT mice and inflammasome-deficient mice exposed to ozone (1 ppm × 3 hr) and phenotyped for airway responsiveness after methacholine challenge (*A*–*C*), cell infiltration (*B*), and BAL protein level (*C*) measured at 24 hr after exposure. (*A*–*C*) caspase1 (*A*), ASC (*B*), and Nlrp3 (*C*) are necessary for the development of ozone-induced AHR. (*B*) Total cells, macrophages, and neutrophils in BALF from ozone-exposed WT mice were higher than those from caspase1-, ASC-, and Nlrp3-deficient mice. (*C*) The level of BAL protein was higher after ozone exposure and was completely dependent on caspase1 and ASC, and partially dependent on Nlrp3. Data are presented as mean ± SE (*n* = 5) and are representative of two similar experiments.
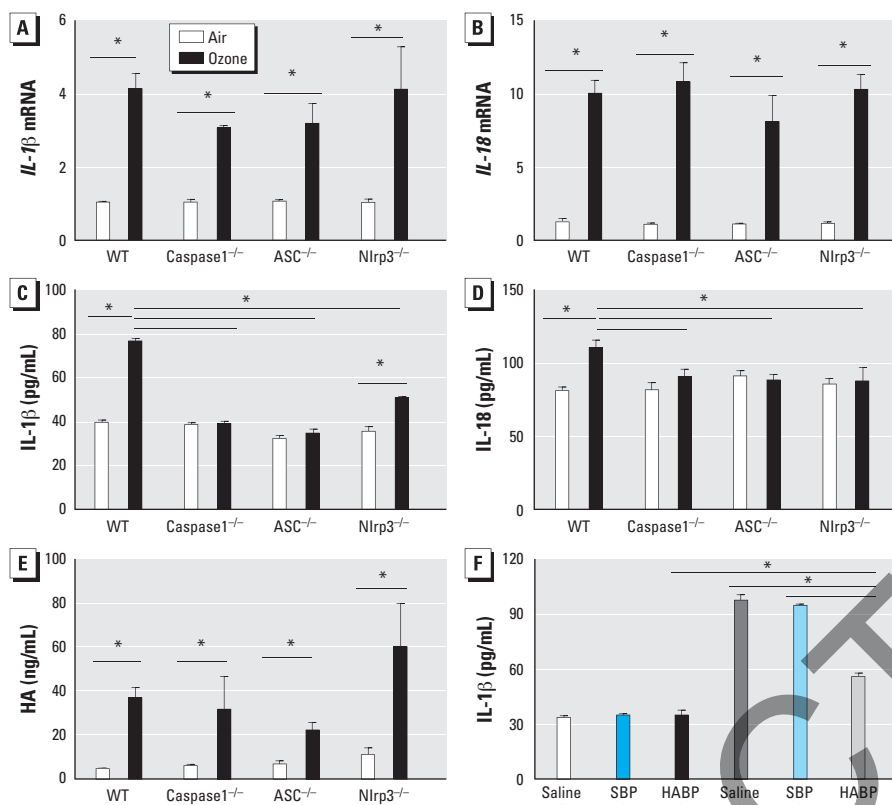*p < 0.05.

HA activation of the Nlrp3 inflammasome results in cleavage of macrophage-derived caspase1 and IL-1β. Activation of the inflammasome requires cleavage of pro-caspase1 to an active form that can subsequently cleave pro-IL-1β to an active soluble form. We therefore determined whether *in vivo* challenge with HA fragments resulted in cleavage of pro-caspase1 in alveolar macrophages. HA resulted in cleavage of pro-caspase1 in WT mice but not in ASC$^{-/-}$, Nlrp3$^{-/-}$, or caspase1$^{-/-}$ mice (Figure 6E). The cleavage of pro-IL-1β is a result of Nlrp3 inflammasome activation. We also observed cleavage of pro-IL-1β in alveolar macrophages from WT mice but not in those from Nlrp3 inflammasome-deficient mice (Figure 6F).

Discussion

We found a dominant role of the Nlrp3 inflammasome in nonallergic reactive airways disease after inhalation of ozone. Development of ozone-induced AHR requires several components of the inflammasome complex, including ASC, caspase1, and Nlrp3. Ozone inhalation results in an increased level of soluble HA, which we previously reported to contribute to AHR (Garantziotis et al. 2009). In the present study, we found that HA-induced cleavage of alveolar macrophage-derived pro-IL-1β is dependent on the Nlrp3 inflammasome. In addition, the levels of several proinflammatory factors (IL-1α, IL-6, KC, TNF-α, MCP-1, IL-17, and C3a) are partially dependent on genes of the Nlrp3 inflammasome after challenge with either ozone or HA. Together these findings strongly support biological and functional roles of the Nlrp3 inflammasome in the development of ozone-induced reactive airways disease.
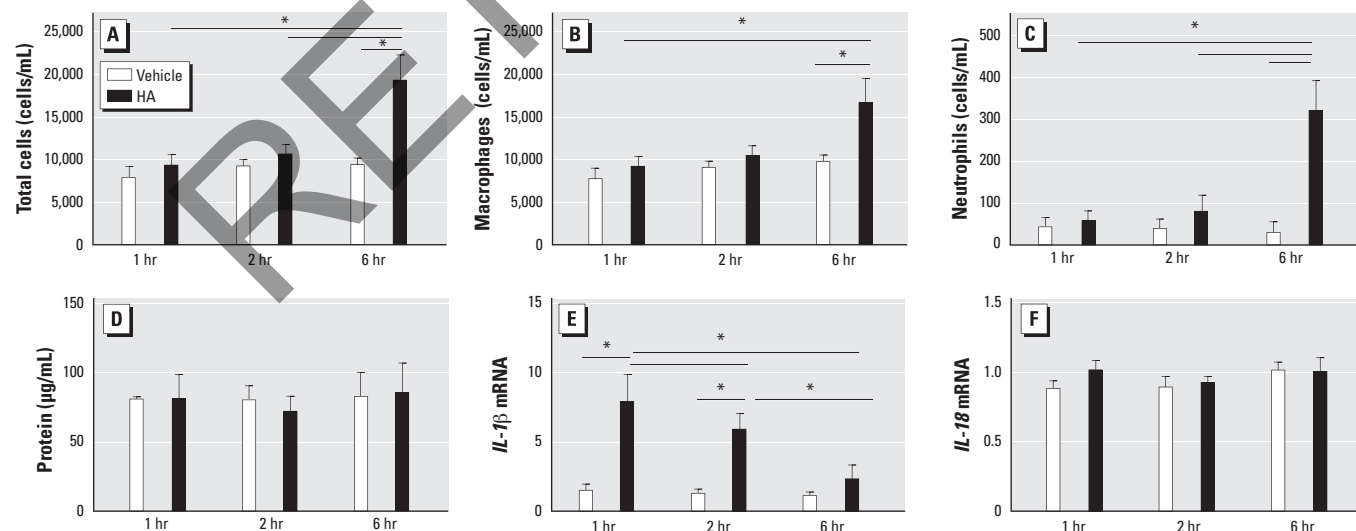
Figure 3. IL-1β, IL-18, and HA in BALF from WT mice and inflammasome-deficient mice 24 hr after exposure to ozone (1 ppm × 3 hr). (*A–B*) The transcription levels of IL-1β (*A*) and IL-18 (*B*) in alveolar macrophages were up-regulated in ozone-exposed mice compared with air-exposed mice, and were not dependent on the Nlrp3 inflammasome. (*C–D*) Increases in IL-1β (*C*) and IL-18 (*D*) proteins were completely dependent on ASC and caspase-1, IL-1β was partially dependent on Nlrp3, and IL-18 was completely dependent on Nlrp3. (*E*) Ozone exposure enhanced the level of soluble HA in BAL; the level of HA was not dependent on Nlrp3 inflammasomes. (*F*) The level of secreted IL-1β in BAL was increased after ozone exposure, but was partially decreased by HABP compared with air, saline, and scrambled-binding peptide (SBP) controls. Data are presented as mean ± SE (*n* = 5) and are representative of two similar experiments. **p* < 0.05.

Figure 4. Time course of biological responses to HA (50 μL; 25 μg/mouse) or vehicle instilled intratracheally into isoflurane-anesthetized WT mice; responses were observed BALF at 1, 2, and 6 hr after HA treatment. (*A–C*) No increases were observed in total cells (*A*), macrophages (*B*), or neutrophils (*B*) until 6 hr after treatment. (*D*) No increases were detected in protein after HA treatment compared with vehicle at these time points. (*E*) *IL-1*β mRNA in alveolar macrophages was up-regulated 1 hr and 2 hr after HA treatment compared with vehicle, but values decreased to near baseline at 6 hr. (*F*) The transcription level of *IL-18* was not significantly different after HA treatment at the three time points. Data are presented as mean ± SE (*n* = 5) and are representative of two similar experiments. **p* < 0.05.

Current evidence supports an important role for IL-1–dependent signaling in the pulmonary response to ozone (Johnston et al. 2007; Park et al. 2004; Verhein et al. 2008; Wu et al. 2008). Our previous work demonstrated that ozone exposure induced the release of HA fragments (Garantziotis et al. 2009, 2010) and led to an increase in soluble IL-1β within the airways (Garantziotis et al. 2010; Li et al. 2011). In a dermal injury model, activation of pro-IL-1β was shown to be regulated by the Nlrp3 inflammasome in response to HA (Yamasaki et al. 2009). In the present study, we found that ASC, caspase1, and Nlrp3 contribute to ozone-induced release of soluble IL-1β.

Although immunostimulatory short fragments of HA can contribute to the production of many macrophage-derived proinflammatory cytokines (Garantziotis et al. 2009, 2010), we have identified the relationship with genes of the Nlrp3 inflammasome. The levels of soluble HA in the airspace and macrophage-derived transcript of *pro-IL-1β* were not dependent on ASC, caspase1, or Nlrp3. However, soluble HA functionally contributed to the generation of active soluble IL-1β after exposure to ozone, as observed in HA-binding experiments. The functional response to direct HA fragment challenge in the lung was dependent on genes of the inflammasome. We observed a dominant role for both ASC and caspase1 in induction of many proinflammatory factors previously associated with the biological response to ozone, including IL-1β, IL-18, IL-1α, IL-6, MCP-1, TNF-α, KC, IL-17, and C3a. These data support that the inflammasome components not only activate IL-1β and IL-18 but also can regulate the release of additional proinflammatory cytokines (Barker et al. 2011). The near complete dependence on caspase1 supports activation of the inflammasome and essentially excludes the contribution of pyronecrosis, which is caspase1 independent but Nlrp3 and ASC dependent (Ting et al. 2008). However, we did not observe consistent complete reduction in any single soluble factor to explain the complete dependence

of the Nlrp3 inflammasome in AHR. This observation suggests that factors other than measured cytokines may contribute to AHR in the Nlrp3-deficient mouse, such as additional soluble factors (Backus et al. 2010; Williams et al. 2008), neural responses

(Caceres et al. 2009), or smooth muscle function. Furthermore, the partial reduction in proinflammatory cytokines observed in Nlrp3-deficient mice suggests that additional proteins in the NLR family may contribute to the response to both ozone and HA.
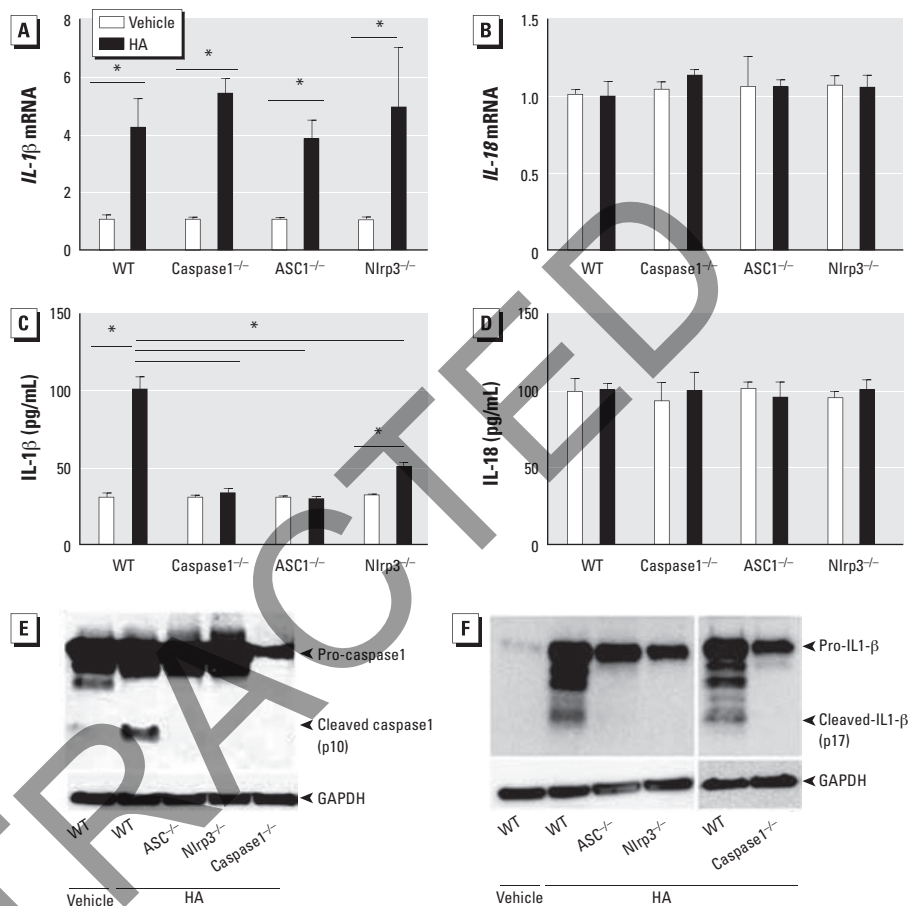

Figure 6. *IL-1β* and *IL-18* mRNA (*A,B*) and protein (*C,D*), cleaved caspase1 (*E*), and cleaved IL-1β (*F*) in alveolar macrophages from WT and inflammasome-deficient mice 2 hr after challenge with short-fragment HA (25 μg/mouse). (*A*) *IL-1β* transcription was increased in alveolar macrophages from HA-treated mice compared with vehicle controls and was not dependent on the Nlrp3 inflammasome; however, *IL-18* mRNA expression (*B*) was not affected by HA. (*C*) After HA treatment, IL-1β in BALF from WT mice was higher than that from Nlrp3 inflammasome-deficient mice. (*D*) The IL-18 protein level was not increased. HA challenge results in cleavage of pro-caspase1 and cleavage of pro-IL-1β in WT mice. (*E,F*) Western blot analysis for cleaved caspase1 (*E*) and cleaved IL-1β (*F*) identified cleavage only in HA-exposed WT mice but not in ASC⁻/⁻, Nlrp3⁻/⁻, or caspase1⁻/⁻ mice. Data are presented as mean ± SE (*n* = 5) and are representative of two similar experiments.
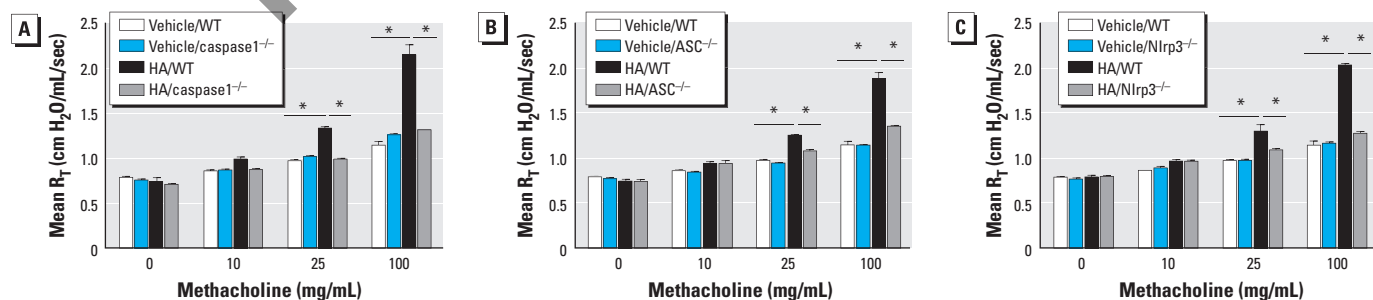*p < 0.05.

Figure 5. The role of the Nlrp3 inflammasome in airway response to HA in WT and inflammasome-deficient mice 2 hr after challenge with short-fragment HA (25 μg/mouse). Compared with WT mice, mice deficient in caspase1 (*A*), ASC (*B*), or Nlrp3 (*C*) were protected from HA-induced AHR. Data are presented as mean ± SE (*n* = 5) and are representative of two similar experiments.
*p < 0.05.

Although ASC and caspase1 are ubiquitously expressed in many cell types, Nlrp3 is primarily expressed in myeloid cells (Guarda et al. 2011). NLR proteins expressed in non-myeloid cells may prove important. However, the partial dependence on Nlrp3 does suggest an important contribution of myeloid-derived activation of the inflammasome for the complete response to either ozone or HA. These findings demonstrate that both ASC and caspase1 play a dominant role in airway hyperresponsiveness and suggest a functional role for the alveolar macrophages as an important source of proinflammatory cytokines.

Current evidence supports an emerging paradigm in which inhalation of ozone results in oxidant-induced fragmentation of HA in the extracellular matrix (Li et al. 2010). These immune-stimulatory fragments of HA interact with a surface receptor complex of CD44–TLR4 (Garantziotis et al. 2010; Taylor et al. 2007) to activate an intracellular signaling cascade that includes MyD88, TIRAP, and NF-κB (Garantziotis et al. 2010; Li et al. 2011), which in turn results in increased mRNA expression of *pro-IL-1*β in alveolar macrophages. Either ozone or HA results in cleavage and activation of pro-caspase1 resulting in activation of the Nlrp3 inflammasome complex. Finally, the activated inflammasome complex results in cleavage of pro-IL-1β into active soluble IL-1β. Our results suggest that active IL-1β contributes to the complex signaling network required for proinflammatory signaling and development of AHR. Together, these new findings provide additional support for a dominant role of genes of innate immunity in nonallergic reactive airways disease. Understanding the mechanisms that contribute to ozone-induced functional phenotypes in the airway can provide fundamental insight into the pathogenesis of AHR, a defining phenotype of clinical asthma. Our results establish a central role for the Nlrp3 inflammasome in both the induction of AHR and production of proinflammatory cytokines. Our observations support that airway therapy targeted toward local deactivation of the Nlrp3 inflammasome may provide benefit to some patients with reactive airways disease.

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Retraction: "Hyaluronan Activation of the Nlrp3 Inflammasome Contributes to the Development of Airway Hyperresponsiveness"

The authors request retraction of "Hyaluronan Activation of the Nlrp3 Inflammasome Contributes to the Development of Airway Hyperresponsiveness." Due to concerns about the initial data from the animal physiology laboratory at Duke, the authors re-exported source data from the Flexivent machine and compared these data with raw data originally received from the animal pulmonary physiology laboratory. Results from these initial comparisons suggested potential inconsistencies in the data. Therefore, the authors proceeded to replicate experiments of animal airway physiology presented in Figure 2A. These replicate experiments failed to validate the originally reported role of Nlrp3, ASC, and caspase 1 in airway hyperresponsiveness after exposure to ozone. These observed inconsistencies significantly impact the overall conclusions of the manuscript. Therefore, all of the authors have agreed to retract this paper in an effort to correct the scientific record. The authors sincerely apologize for any inconvenience to the scientific community.

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